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(54) Title: RAPID ASSAY TO ESTIMATE GENE TRANSFER INTO CELLS

(57) Abstract

A rapid and quantitative assay to estimate gene transfer into cells using a 96-well format PCR is described. The assay can be combined with a fluorescent detection system to eliminate the need to run agarose gels. An assay and primers are provided for detection of Moloney-Murine leukemia virus (MMLV)-based vectors.

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RAPID ASSAY TO ESTIMATE GENE TRANSFER INTO CELLS

FIELD OF THE INVENTION

This invention relates to assays for detecting gene transfer into cells, for example, determining gene transfer into cells transfected with a known vector such as a Moloney-Murine leukemia virus (MMLV)-based vector.

BACKGROUND OF THE INVENTION

Retrovirus vectors are used to achieve stable, long-term expression of a foreign gene in a host cell. Commonly utilized retroviral vectors include Moloney murine leukemia virus (MMLV), Harvey murine sarcoma virus, murine mammary tumor virus, and Rous Sarcoma virus. These vectors can transfer or include a sequence for a selectable marker so that transduced cells may be identified and generated. Generally, the gene of interest is cloned into a retrovirus vector that lacks most viral genes. The gene is usually expressed under the control of the strong viral promoter in the LTR. The recombinant plasmid is transfected into a special packaging cell line that harbors an integrated provirus. Packaging cell lines include, for example, Ψ2, PA317, and PA12. The provirus is designed such that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into a virus. Thus, the packaging cells produce empty virions, since no genome is packaged. If a retroviral vector containing a Ψ packaging sequence is introduced into the packaging cells, RNA produced from the recombinant virus is packaged into infectious viral particles. The virus stock released from the packaging cells thus contain only the recombinant virus. The virus can be used to infect a

host cell, resulting in the integration of the viral genome and the stable production of the foreign gene product (Armentano et al. (1987) J. Virol. 61:1647-1650; Bender et al. (1987) J. Virol. 61:1639-1646).

The polymerase chain reaction (PCR) is a useful method for enzymatically amplifying nucleic acid sequences (See, e.g., Mullis, U.S. Patent No. 4,683,202). The basic principle of PCR is the exponential replication of a DNA sequence by successive cycles of primer extension. The extension product of one primer, when hybridized to another primer, becomes a template for the synthesis of another nucleic acid molecule. The primer template complexes act as substrate for DNA polymerase which, in performing its replication function, extends the primers. The region in common with both primer extensions, upon denaturation, serves as a template for thermostable DNA polymerases, e.g., such as that isolated from *Thermus aquaticus* or Taq DNA polymerase. Other amplification methods currently under development include LCR (ligase chain reaction, BioTechnica International) which utilizes DNA ligase, and a probe consisting of two halves of a DNA segment that is complementary to the sequence of the DNA to be amplified; enzyme QB replicase (Gene-Trak Systems) and a ribonucleic acid (RNA) sequence template attached to a probe complementary to the DNA to be copied which is used to make a DNA template for exponential production of complementary RNA; and NASBA (nucleic acid sequence-based amplification, Cangene Corporation) which can be performed on RNA or DNA as the nucleic acid sequence to be amplified (Gemen et al. (1993) AIDS 7:5107-5110).

PCR is an extremely sensitive assay which has many uses in retroviral-mediated gene transfer protocols (Morgan et al. (1990) Human Gene Therapy 1:135). PCR is routinely used to estimate gene transfer following

retroviral transduction of cells. Typically, the PCR target is specific to the introduced gene, i.e., the presence of an introduced marker or therapeutic gene is used to detect transduction.

BRIEF SUMMARY OF THE INVENTION

The present invention provides rapid and sensitive gene transfer assays applicable to gene transfers accomplished without regard to the specific vector used. One non-limiting example of the invention provides an assay for the detection of gene transfer by Moloney-Murine leukemia virus (MMLV)-based vectors.

Thus, in one aspect, the invention is directed to a method for detecting gene transfer by a vector which includes a gene and a sequence unique to that vector, such as a MMLV-based vector, by (1) obtaining a population of cells, (2) lysing the cells to obtain a cell lysate, (3) contacting the cell lysate with a pair of oligonucleotide primers corresponding to a segment of unique sequence, e.g. unique MMLV DNA, under conditions in which a DNA segment complementary to the primers is amplified, and (4) detecting the amplified DNA sequence, which is indicative of gene transfer.

As shown below, the method of the invention is sufficiently sensitive to detect gene transfer in a cell population having a single transduced cell in a population of cells. In one embodiment, the method is used to detect gene transfer in a single cell colony. However, the method may also be used to detect gene transfer in a bulk cell population including transduced and non-transduced cells. In a specific embodiment of the invention, a colony of hematopoietic stem/progenitor cells is analyzed for gene transfer.

The oligonucleotide primer pair used for detection amplifies a sequence unique to a retroviral vector

packaging gene and not present in the host cells, preferably the PCR amplifies a packaging sequence conserved in MMLV-based vectors. In a preferred embodiment, the packaging sequence is the Ψ packaging sequence. In a specific embodiment, the oligonucleotide primers amplifying the Ψ packaging sequence are selected from the primers having the sequences of SEQ ID NO:1-8. More preferably, the primers used are those having the sequence SEQ ID NO:1/SEQ ID NO:8 and SEQ ID NO:2/SEQ ID NO:8. Most preferably, the primers used have the sequence of SEQ ID NO:1 and SEQ ID NO:8.

In one embodiment, the method of the invention is used with more than one pair of oligonucleotide primers. In addition to the oligonucleotide primers corresponding to a segment of MMLV DNA, a second pair of primers may be used which correspond to a second marker of interest. In a specific embodiment, the second pair of primers correspond to β-globin DNA as an internal control for the PCR reaction. The use of multiple primer pairs to other markers of interest are included in the method of the invention.

The amplified PCR product may be detected in a number of ways, including by gel electrophoresis and Southern blots. The amplified PCR product may also be detected by incorporation of a label into the PCR product, including radioactive, fluorescent, chemiluminescent, etc. In one embodiment of the method of the invention, the amplified PCR product is measured by the amount of fluorescence generated in the PCR reaction mixture. In this embodiment, a fluorescent oligonucleotide probe complementary to a segment of the amplified PCR product is included with the first primer pair, and the level of fluorescence in the PCR reaction mixture is proportional to the concentration of amplified PCR product.

In another aspect, the invention features oligonucleotide primers useful in the method of the invention for detection of a MMLV-based retroviral vector transduced cell. The primers of the invention include those shown in Table 1 (SEQ ID NO:1-8). Preferred primers are those having the sequence of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:8. The most preferred primer pair sequence are those having the sequence of SEQ ID NO:1 and SEQ ID NO:8.

In one aspect, the invention features a kit for determining gene transfer in a cell, providing buffers and primers suitable for performing the assay of the invention. In one embodiment, a kit for the analysis of transduction frequency in single methylcellulose colonies includes a 96-well plate containing PCR lysing buffer, PCR reagents (sterile water, 10 x buffer, dNTPs, a pair of PCR primers, polymerase, and optionally, control primers such as β-globin primers), detection reagents (probe, either fluorescently labeled or ^{32}P-ATP labeled), and instructions. In another embodiment, a kit for the estimation of transduction frequency in a bulk assay contains bulk standards (premade cell lysates), PCR reagents as above, and instructions for use.

The assay of the present invention is convenient and simple, and it is universal in that it can be used without regard to the specific gene being transferred, thus lending it to formulation in kits without the need to develop unique primers and to optimize conditions for use of those primers. For example, it is not necessary to re-define the conditions for maximum sensitivity, and specificity of the PCR by optimization of the various different reaction parameters, such as primer concentration, annealing temperature, salt concentration, and magnesium ion concentration (*e.g.*, KCl, $MgCl_2$) (Saiki (1989) in PCR Technology: Principles and Applications for

DNA Amplification (Erlich, H.A., ed.); M. Stockton Press, NY; p. 7).

Other aspects, features, and advantages of the invention will become apparent from the following detailed description, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a sequence comparison of the extended packaging signal (Ψ) of several Moloney-based retroviruses. A "•" indicates that the nucleotide is unchanged from the Moloney sequence, and a "-" indicates that a nucleotide has been deleted. Nucleotide substitutions are as indicated. Both the upstream primer (F1) and the downstream primer (B5) are underlined.

Fig. 2A is an ethidium bromide stained agarose gel showing a representative XP outcome (excessive non-specific bands) obtained with primers F2/B1. Lanes 1, 2, 3, and 4 are the result of amplifying 250 cell equivalents of β7 (Ψ positive), KG-1a (Ψ negative), SV29 (Ψ positive), and Sp6-γ (Ψ positive) cells; "-" is a negative PCR buffer control. An arrow indicates the size of the expected PCR product based on the sequence of the MMLV sequence (see Fig.1).

Fig. 2B is an ethidium bromide stained agarose gel showing a representative IP outcome (insufficient PCR product formation) obtained with primers F2/B4. Lanes 1, 2, 3, and 4 are as described in the legend to Fig. 2A.

Fig. 2C is an ethidium bromide stained agarose gel showing a representative NP outcome (no PCR product formation) with LXSN/LNL6-based vectors F3/B1. Lanes 1, 2, 3, and 4 are as described in the legend to Fig. 2A.

Fig. 2D is an ethidium bromide stained agarose gel showing a representative OK outcome (PCR product of the expected size with little or no non-specific bands)

obtained with vectors F1/B5. Lanes 1, 2, 3, and 4 are as described in the legend to Fig. 2A.

Fig. 3A is an ethidium bromide stained agarose gel showing the absolute sensitivity of the primers F1/B5 in a 40 cycle PCR. The primer pair was tested against a 2-fold dilution series of β7 (Ψ positive) cell lysates at 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 cell equivalents. "-" is a negative PCR buffer control; "L" is a 100 bp ladder (500, 400, 300, 200, 100, 50 bp; an arrow indicates the position of the 134 bp Ψ PCR product.

Fig. 3B is an ethidium bromide stained agarose gel showing the detection capability of the F1/B5 Ψ primers in a 30 cycle bulk PCR assay. Bulk PCR controls were made by adding KG-1a cells (Ψ negative) to a two-fold dilution series of SV29 (Ψ positive) cells. Lysates "A" through "F" contain 10, 5, 2.5, 1.3, 0.6, and 0.3% SV29 cells, respectively. Lysate "$\bar{}$" contains 100% KG-1a cells. The total number of cell equivalents per PCR is 2,500. "L" is a 100 bp ladder (500, 400, 300, 200, 100, 50 bp); an arrow indicates the position of the 134 bp Ψ PCR product.

Fig. 4A is an ethidium bromide stained agarose gel of β7 (Ψ positive) and KG-1a (Ψ negative) colonies pooled and processed for the PCR having a ratio of 4:1, 3:2, and 2:3 KG-1a:β7 colonies.

Fig. 4B is an ethidium bromide stained agarose gel showing the effect of residual methylcellulose medium on PCR sensitivity in a lysate consisting of β7 cells in a background of KG-1a cells. Samples in lanes 1-6 all contain 375 KG-1a cell equivalents. Samples in lanes 1 & 4, 2 & 5, and 3 & 6 contain 125, 62.5, and 12.5 β7 cell equivalents, respectively. Samples in lanes 4-6 were processed with the addition of methylcellulose medium, while samples in lanes 1-3 were processed without the addition of methylcellulose medium.

Fig. 5A is an ethidium bromide stained agarose gel of direct lysate PCR from single methylcellulose colonies of β7 (Ψ positive) and KG-1a (Ψ negative) cells using only the Ψ primers F1/B5 (top row), or the Ψ and β-globin primers (multiplex PCR - bottom row). An arrow indicates the position of the 134 bp Ψ PCR product and/or the 250 bp β-globin product. "Ψ Buffer" is a negative PCR control of the buffer used to process the colonies, and "L" is a 100 bp ladder.

Fig. 5B is a Southern blot of the agarose gel of Fig. 5A hybridized to the Ψ PCR product internal probe, BK29.

Fig. 6A is an ethidium bromide stained agarose gel of eighty 14-day methylcellulose colonies derived from hematopoietic stem/progenitor cells growing in methylcellulose medium which were harvested and directly lysed in the 96-well format; a sample of the lysate from each colony was amplified using the F1/B5 Ψ primers (#'s 1-80), positive colonies are numbered above the lane; also amplified in the same plate were 10 hematopoietic colonies of Mock transduced cells ("Mocks"); positive controls, (SV29) ("+"); and negative PCR controls: "-" (PCR master mix without lysing buffer; "Buf" (PCR master mix with lysing buffer); "L" is an 100 bp ladder, and "+" is a positive control (134 bp band) loaded on each side of the gel. For each panel an arrow indicates the location of the F1/B5 Ψ PCR product.

Fig. 6B is the Southern blot of the agarose gel described in Fig. 6A above, hybridized with the internal probe BK29 (B).

Fig. 7A is a representation of the results obtained by ethidium bromide staining (slanted bars) and fluorescence (solid circle) in a 96 well plate. Samples #1-80 represent colonies derived from hematopoietic stem/progenitor cells exposed to retroviral supernatant,

samples #81-90 are colonies of Mock-transduced cells, "+" represents single SV29 (Ψ positive) colonies, "KG-1a" is a single Ψ negative colony, and "Buffer" is a negative PCR control.

Fig. 7B is a graph showing the results of a direct comparison of an ethidium bromide stained gel and the TaqMan™ assay for a subset of the samples of Fig. 7A (#55-90). The dashed line represents the threshold ΔRQ above which samples are positive at the 99.95% confidence level. TaqMan™ positive samples are indicated by shaded bars.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features a rapid and highly sensitive assay for detecting gene transfer into cells, for example, Moloney-Murine leukemia virus (MMLV)-based gene transfer. The assay of the present invention is widely applicable since the majority of retroviral vectors used in current gene transfer protocols are MMLV-based (Jolly (1994) Cancer Gene Therapy 1:51) and the assay is specific to all known MMLV-based vectors.

Before the methods of the invention are described, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "a cell" or "a cell colony" include mixtures of such cells or cell colonies.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as

commonly understood by one of ordinary skill in the art to which the invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of disclosing and describing the material for which the reference was cited.

Definitions

By **"gene transfer"** is meant the insertion of a exogenous fragment of nucleic acid into a cell and the subsequent recombination of part or all of the exogenous DNA into the cell's genome. Gene transfer includes the insertion of one or more exogenous nucleotide sequences into a cell by transduction or transfection.

The nucleic acid transferred may include all or part of a gene of interest, for example, a nucleic acid sequence encoding a protein or a therapeutic gene. For example, the nucleic acid transferred into a cell may be a therapeutic gene which confers resistance to chemotherapeutic agent, thereby protecting the cells and their progeny from chemotherapeutic treatment. Similarly, a therapeutic gene may be transferred which confers resistance to an infectious agent. Therapeutic genes also includes antisense or ribozyme sequences, which interfere with viral infection or replication in the transformed cell. Alternatively, the introduced nucleic acid sequences may serve as "decoys", e.g., products which bind essential viral proteins, thereby interfering with the normal viral life cycle and inhibiting replication.

By **"detection"** of gene transfer is meant a method which is able to determine the presence of an exogenous fragment of DNA in a cell. Means of detection include amplification of the specific DNA fragment of interest, e.g., MMLV DNA, and visualization or quantitation of the exogenous DNA fragment by gel electrophoresis, or by

labelling of the amplified PCR product, e.g., with radioactivity, fluorescence, chemiluminescence, etc.

By **"amplification"** is meant any method by which a specific segment of nucleic acid is preferentially synthesized in a detectable amount. In the illustrated method of the invention, PCR is used to amplify a segment of DNA complementary to the vector Ψ packaging sequence. The method of the invention may also be used with other methods of amplification currently available or under development. Amplification methods suitable for use in the method of the invention include the following.

By **"packaging sequence"** is meant a gene or fragment of a gene encoding proteins required for packaging of viral RNA into infectious virus particles. Packaging sequences include a wide number of vector-specific packaging genes and portions of genes known to the art. The packaging sequences useful in the invention include genes and gene fragments of retroviral packaging systems, such as the PA12, Ψam, PA317, ΨCRIP, DAN, HX, Gibbon Ape Leukemia-based, VSVg, and kat systems (Jolly (1994) Cancer Gene Therapy 1:51-64). A preferred packaging nucleotide sequence is a portion of the MMLV Ψ packaging sequence.

PCR. The PCR exploits certain features of DNA replication to amplify a selected nucleic acid segment. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand; reverse transcriptase uses RNA as a template to produce a complementary new strand (cDNA). These single-stranded DNA templates can be produced by simply heating double-stranded nucleic acids to temperatures near boiling. A small section of double-stranded template is required to initiate ("prime") synthesis. Therefore, the starting point for nucleic acid synthesis can be specified by supplying an oligonucleotide primer that anneals to the

template at that point. Thus, the DNA polymerase can be directed to synthesize a specific region of DNA.

Both DNA or RNA strands can serve as templates for synthesis provided an oligonucleotide primer is supplied for each strand. For a PCR, a pair of primers are chosen to flank a region of the nucleic acid segment to be amplified so that the newly synthesized strands of DNA, starting at each primer, extend beyond the position of the primer on the opposite strand. Therefore, new primer binding sites are generated on each newly synthesized DNA strand. The reaction mixture is again heated to separate the original and newly synthesized strands, which are then available for further cycles of primer hybridization, DNA synthesis, and strand separation. The net result of a PCR is that by the end of n cycles, the reaction contains a theoretical maximum of 2^n double-stranded DNA molecules that are copies of the DNA sequence between the primers, i.e., amplification of the selected DNA sequence.

Assay for MMLV-based Gene Transfer

The methods of the present invention may be used to develop assays universal for any vector used to deliver exogenous genetic sequences to a target cell. Thus, the methods of the present invention may be used to develop assays universal to any vector including, for example, adenovirus vectors, adeno-associated virus vectors, MMLV vectors, Harvey sarcoma virus (HSV) vectors, and the like. Preferably, the assay is specific for MMLV vectors.

In order to develop an assay useful in the detection of gene transfer by MMLV-based vectors, the MMLV provirus genome was examined for regions which would be found in most or all MMLV-based vectors, and which would not be found in the target cell genome. Any

vector-specific sequence may be used in the present invention as long as it is retained in the vector backbone of any given retroviral vector, and its amplification by PCR does not concomitantly amplify sequences from the host genome of transduced cells. Thus, it is desirable to use as target sequences for amplification, vector sequences required for gene delivery or expression and, therefore, not likely to be replaced or modified in any given vector.

The Ψ packaging sequence is a 300-350 bp region downstream of the 5′ LTR which extends into the *gag* coding sequence (Bender et al. (1987) J. Virol. 61:1639). This region is required for efficient packaging of retroviral genomic RNA molecules. In addition, complete inclusion of this region in retroviral vectors has been shown to increase viral titer 5-10 fold relative to retroviral vectors which contain 3′ deletions in this region (Armentano et al. (1987) J. Virol. 16:1647). Alternative vector sequences may be used in the present invention, including LTRs and tRNA primer binding sites, although care must be taken to ensure the sequences are universal to all known vectors since some of these regions are modified occasionally in different vector backbones. LTR sequences are less desirable for use in the present invention since there appear to be many similar sequences within the human genome. Preferably, the sequences detected in the present invention are from the Ψ packaging region.

Eight PCR primers that amplify regions of the Ψ packaging sequence from various MMLV retroviral vectors were selected (shown in Table 1) (SEQ ID NO:1-8), and candidate primer combinations were tested in exploratory PCR analyses to identify an ideal universal primer pair (Example 1). Ideally, a primer pair is designed to be universal for all retroviral backbones, producing a band

of the expected size with little or no spurious background bands; by comparison, non-transduced cells should be free of the expected PCR product. To analyze this, multiple primer combinations are designed to amplify the vector sequence of interest. Each primer combination is used, in separate PCR reactions, to amplify DNA sequences from cells, typically human cells having no vector sequences, to test for non-specific background bands. Each primer combination is also tested in PCR reactions with cells carrying different vector backbones to test the universality of the primer pairs. Sensitivity of potential primer pairs is tested in mixtures of cells having different proportions of vector-positive and vector-negative cells.

The primer combination F1/B5 (SEQ ID NO:1/SEQ ID NO:8) was found to meet the criteria for a universal primer pair for MMLV-based retroviral vectors. The F2/B5 primer pair was also shown to meet the criteria of amplifying all MMLV-based vectors tested without spurious background bands. Sensitivity and detection level studies (Example 2) established that the F1/B5 Ψ primer combination could routinely detect transduction efficiencies of down to 0.6% positive cells in a semi-quantitative assay (2,500 cells total).

The primers identified by the methods of the present invention can be used to detect gene transfer into target cells of any tissue type. Typically, the assays will be used to detect gene transfer into human cells. Many primary human cell types are targets for gene therapy and can be assayed in the present invention including hematopoietic cells, neural cells, fibroblasts, liver cells, and tumor cells. Typically, the vector backbone sequences will not be expressed in the transduced cells and, therefore, the integrated DNA sequences will be detected by DNA PCR.

The PCR assay may be used to detect vector sequences in the transduced cells anytime from immediately after the transduction to several days or weeks after the transduction, as long as the cells are viable, or indefinitely if the cells are cryopreserved. Transduction may be assessed by PCR on a bulk cell population after transduction or on colonies grown from single cells. For retroviral vectors it is preferable to allow the target cells to cycle, allowing integration of the retroviral vector into the host cell DNA, permitting a more accurate measure of transduction frequency.

To assess transduction frequency in hematopoietic cells, a sample portion of the transduced cell population may be plated in a semi-solid media such as methylcellulose. The resulting colonies, each representing single cells from the original transduction mix, can be picked and either pooled or analyzed individually for presence of the vector sequences.

To demonstrate the efficacy of the Ψ primers in detecting transduced methylcellulose colonies when these colonies are pooled, 5 colonies growing in methylcellulose were pooled and processed for the PCR (Example 3). Three different lysates were analyzed which had different ratios of non-transduced and transduced colonies: 4:1, 3:2, and 2:3. The data presented in Fig. 4A demonstrates that a single transduced colony is detectable even when present in a background of 4 non-transduced colonies, indicating that the assay has sufficient sensitivity to be effective when pooling colonies is required, for example, under conditions where low transduction efficiencies are expected.

There has been some concern that trace amounts of methylcellulose medium, which can contaminate the lysate during the processing of hematopoietic stem/progenitor cells, can interfere with the PCR (Bregni et al. (1992)

J. Clin. Invest. 82:1017). Residual methylcellulose medium can reduce the sensitivity of the PCR when using alternative primer sites to detect transduction (e.g., *lacZ*, neomycin targets; data not shown). An experiment was conducted to determine the effect of residual methylcellulose media on Ψ PCR sensitivity in a lysate consisting of β7 cells (Ψ positive) in a background of KG-1a cells (Ψ negative) (Example 3). The results, shown in Fig. 4B, indicate that using the primer sequences F1 and B5 demonstrates no significant loss of PCR sensitivity and/or specificity attributable to trace amounts of methylcellulose in the PCR. These results are particularly relevant when using the single-colony direct lysate PCR technique presented below.

Example 4 describes a single colony direct lysis PCR protocol which can be used to detect transduced human cells in clonogenic methylcellulose assays. Single colonies of clonogenic cells growing in methylcellulose medium (Brandt et al. (L1988) J. Clin. Invest. 82:1017) are routinely analyzed by PCR (Dick et al. (1991) Blood 78:624). Cassel et al. (1993) Exp. Hematol. 21:585 have used a direct lysis protocol where single colonies are picked and lysed in H_2O prior to PCR analysis. However, most single colony protocols involve multiple rinsing steps (Lu et al. (1993) J. Exp. Med. 178:2089; Ramaswamy et al. (1989) Exp. Hematol. 17:832; Rill et al. (1992) Human Gene Therapy 3:129) and even DNA extractions (Lu et al. (1994) Human Gene Therapy 5:203). The single-colony direct lysate technique described here involves no rinsing of the colonies, and the lysate is used directly in the PCR reaction without protein extraction or DNA precipitation. PCR was performed with Ψ primers or a mix of Ψ and β-globin primers as an internal control. All β7 and SV29 colonies were positive for both Ψ and β-globin, and KG-1a colonies were positive only for β-globin.

Positive colonies detected by ethidium bromide stained agarose gels were confirmed by Southern blot analysis (Figs. 5A and 5B).

Example 5 describes the determination of gene transfer in transduced human $CD34^+$ $Thy\text{-}1^+$ Lin^- hematopoietic stem cells isolated from cytokine mobilized human peripheral blood. The cells were sorted (Becton Dickinson, FACStar Plus), transduced with a Moloney-based LMTNL retroviral vector, and plated in methylcellulose medium to assess transduction of the stem/progenitor cell population. Eighty 14-day methylcellulose colonies were harvested individually and lysed in the 96-well format; a sample of the lysate from each colony was amplified using the F1/B5 Ψ primers. Also amplified in the same plate were 10 hematopoietic colonies of Mock transduced cells, i.e., no retroviral vector, positive controls (SV29, Ψ positive colonies), and negative PCR controls. Fig. 6A shows the positive samples detected by ethidium bromide staining. Southern blots of the positive samples probed with the internal probe BK29 (shown in Fig. 6B) verified the specificity of the Ψ positive samples.

The direct lysate technique described in Example 5 permits the effective processing of a large number of samples in the conventional and convenient 96-well format. An important advantage of single-colony direct lysate PCR analysis carried out in a 96-well format is the reduction in processing time. Methylcellulose colonies are harvested directly into the PCR lysing buffer, thereby omitting the PBS rinse step. Decreased handling of the lysate further reduces the potential for contamination and sampling error due to multiple pipetting steps. These time-saving steps allow processing of greater numbers of samples, while utilizing the 96-well format permits the use of multichannel pipetters to expedite the addition of reagents, lysates,

etc. Furthermore, many popular thermal cyclers with 96-well formats easily accommodate small PCR reaction volumes, leading to a substantial decrease in the cost of reagents.

Aside from expediting the PCR set-up, the single-colony direct lysate technique yields a more accurate estimate of transduction frequency than prior art methods. When colonies are pooled, calculating the transduction frequency will almost always be an underestimate since when a sample is PCR positive one can never be absolutely certain if single or multiple colonies were transduced. In addition, pooling 5 colonies for PCR analysis frequently results in taking up 10-12 μl of methylcellulose media. Large volumes of methylcellulose may not only interfere with the PCR, but increase the odds of inadvertently collecting stray cells which are not part of the colony being harvested. These stray cells could lead to detectable levels of PCR product resulting from cells which are not clonogenic in methylcellulose. Single-colony direct lysis PCR in a 96-well format makes it practical to screen a large number of colonies without the need for pooling. Furthermore, when single methylcellulose colonies are picked for PCR analysis, the transduction efficiency of specific colony types can be more readily determined.

The PCR products of the present invention may be detected by any method known in the art including, for example, by ethidium bromide gel electrophoresis and/or Southern blot analysis. However, for the high-throughput laboratory, it can become tedious to examine the PCR product of a large number of samples using a gel-based ethidium bromide-staining detection system. It follows that an alternative detection system such as a fluorescence-based post-PCR detection assay, which can take advantage of the 96-well format of the PCR, would be

a step forward in expediting an already improved and efficient assay.

For example, the TaqMan™ fluorescent system for post-PCR analysis was found to be fast, convenient, and as sensitive as an ethidium-bromide based detection system, as determined by comparison with detection of the PCR product amplified with the F1/B5 Ψ primers (Example 6). Furthermore, the specificity of the internal probe makes a hybridization assay of PCR product unnecessary. The TaqMan™ system of PCR product detection uses the 5′→3′ exonuclease activity of *Thermus aquaticus* DNA polymerase (Holland et al. (1991) Proc. Natl. Acad. Sci. USA 88:7276) to cleave a fluorescently labeled probe during each round of PCR amplification (Lee et al. (1993) Nuc. Acids Res. 21:3761). Post-PCR product analysis merely involves placing the 96-well tray/retainer assembly containing the open PCR reaction tubes into the specially designed fluorescent plate reader, and within minutes, the spectrophotometer reads the fluorescence signal from all 96 wells and summarizes the results in spreadsheet format. Since the assay does not consume any part of the reaction volume, the samples can be saved and used for other purposes (e.g., gel verification). It is theoretically possible that PCR reaction tubes be designed such that the fluorescence can be read directly through the cap, in which case, data acquisition would not require opening the PCR reaction tube, and the potential for PCR product contamination of subsequent amplifications would be significantly reduced.

In general, the detection capability of the TaqMan™ assay depends largely on the design of the fluorescently-labeled probe and the efficient amplification of the target DNA with the PCR primer pair. Guidelines for designing an appropriate fluorescently-labeled probe are provided in the manufacturer's

instructions (Perkin-Elmer). For the amplification of MMLV Ψ sequences herein described, the internal probe is preferably that identified as SEQ ID NO:11. Using the F1/B5 primers and the TaqMan™ probe designed for the Ψ PCR product, a comparison of the ethidium bromide staining detection capability versus TaqMan™ fluorescence from a total of 4 separate amplifications (full 96-well plates, as above; data not shown) demonstrated that a TaqMan™ positive sample (99.95% confidence level) was found in all cases where there was at least a faint ethidium bromide-stained band.

The following examples are meant to illustrate, but not limit, the invention.

Example 1. Materials and Methods.

Ψ Universal Primers. Potential primer sites for the Ψ packaging signal were chosen based on: 1) sequence homology across several MMLV-based retroviruses (see Fig. 1), 2) lack of secondary structure, 3) 40-60% GC content, and 4) melting temperature appropriate for a PCR primer (Rychlik (1993) in: Methods in Molecular Biology, Vol. 15: PCR Protocols, Current Methods and Applications (White B.A. (ed.); Humana Press, Inc., Totowa, NJ; p. 31). Melting temperature (T_M) was estimated using the following algorithm: °C = (4)(number of G/C bases) + (2)(number of A/T bases). Table 1 shows the sequence and position of the 8 candidate Ψ primers selected for testing - 3 upstream and 5 downstream oligonucleotides. Their position is based on the Ψ extended packaging signal of the MMLV (Fig. 1). All sequences are referenced according to the sense strand.

TABLE 1. Candidate PCR Primers Used to Amplify a Region in the Ψ Packaging Signal.

PCR Primers	Sequence (5′→3′)	Position	T_M (°C)	SEQ ID NO:
Upstream (sense)				
F1	CgCAACCCTgggAgACgTCC	31-50	68	1
F2	gAACACCCggCCgCAACCCTg	20-40	72	2
F3	gCCCgACCTgAgTCCAAAAATC	77-98	68	3
Downstream (anti-sense)				
B1	ggCgCggCTTCggTCCCAAAC	209-229	72	4
B2	gCAgCAgACAAgACgCgCggC	227-247	72	5
B3	CCTACCAgAACCACATATCCCTCC	136-159	74	6
B4	CAAAAATTCAgACggAggCggg	181-202	68	7
B5	CgTCTCCTACCAgAACCACATATCC	140-164	76	8

Cell Lines. The human acute myelogenous leukemia $CD34^+$ cell line, KG-1a (Koeffler et al. (1980) Blood 56:265), was used as a negative control for the Ψ primers. The KG-1a/β7 cell line (hereafter referred to as "β7") is a KG-1a clone transduced with MFG-*lacZ* and selected by the FACS-Gal procedure (Nolan et al. (1988) Proc. Natl. Acad. Sci. USA 85:2603). The KG-1a/SV29 cell line (hereafter referred to as "SV29") is a KG-1a clone transduced with the MMLV-SV-NLS-*lacZ* retrovirus which was also cloned via FACS-Gal (Bonnerot et al. (1987) Proc. Natl. Acad. Sci. USA 84:6795). The Sp6-γ cell line (Hwu et al. (1993) J. Exp. Med. 178:361) is a producer cell line (PA317) containing the LXSN retroviral vector (Miller & Rosman (1989) BioTechniques 7:980) which is a derivative of the LNL6 retroviral vector (Bender et al. (1987) supra).

PCR Conditions. All PCRs were conducted in a total volume of 25-28 μl using the Perkin Elmer/Applied Biosystems 9600 thermal cycler. Unless otherwise stated, the 40 cycle PCR is as follows: initial denaturation of

30 seconds at 95°C followed by 40 cycles of 10 second denaturation at 95°C, 15 seconds annealing at 64°C, and 15 seconds extension at 72°C. The protocol finishes with a final 5 minute extension at 72°C and a 4°C soak. PCR product was visualized by loading 15 μl on a 4% NuSieve 3:1 agarose gel (FMC Bioproducts) containing ethidium bromide (Sigma). Images were captured with either a Polaroid camera or the Eagle Eye II Still Video System (Stratagene).

The exploratory PCR analyses of potential Ψ primer combinations were conducted using an annealing temperature of 55°C under the following conditions: 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Boehringer Mannheim), 0.35 μM Ψ primers, and 1.0 Units/25 μl reaction of AmpliTaq DNA polymerase (Perkin Elmer/Applied Biosystems. Optimal PCR buffer conditions were determined using the Opti-prime PCR optimization kit (Stratagene). In addition, optimum primer concentration was determined by standard titration experiments. The final concentration of the PCR components for the optimized conditions are as follows:
10 mM Tris (pH 9.2), 75 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Boehringer Mannheim), 0.20 μM Ψ primers, 0.5 μM T4 gene 32 protein (Boehringer Mannheim), and 1.0 Units/25 μl reaction of AmpliTaq DNA polymerase. With the exception of the Ψ primers used in the initial matrix analysis, all PCR primers were HPLC purified. PCR cell lysing buffer consists of 1X PCR buffer (as above) with the addition of 0.5% NP-40 (Sigma), 0.5% Tween-20 (Sigma), and 0.91 mg/ml of proteinase K (Boehringer Mannheim).

Results. A complete matrix of 15 possible primer combinations were investigated for their ability to amplify Ψ packaging sequence DNA. Results are summarized in Table 2 and representative examples of each possible

outcome are shown in Figs. 2A-2D: XP, excessive non-specific product formation; IP, insufficient product formation; NP, no product with an LNL6-based vector; and OK, specific product formation of the expected size, with little or no non-specific products. In each figure, lanes 1-4 represent 250 cell equivalents of the five cell lysates as described above: β7 (Ψ positive), KG-1a (Ψ negative), SV29 (Ψ positive) and Sp6-γ (Ψ positive), respectively; "-" is a negative PCR buffer control. This initial PCR was conducted at an annealing temperature of 55°C in order to permit maximum primer-template annealing.

Many of the primer combinations tested produced a high degree of non-specific bands, potentially the result of the low annealing temperature. However, even with the less stringent conditions of this initial PCR, it was evident that several of the combinations, specifically F1/B5 and F2/B5 were good candidates for a universal primer pair. The PCR product resulting from the Sp6-γ cell lysate (lane 4, Figs. 2A-2D) was slightly smaller in size than the similarly targeted region in the β7 or SV29 cell lines (115 bp versus 134 bp, respectively). The smaller product is a consequence of a number of small deletions in the amplified region of the LNL6/LXSN backbone (see Fig. 1).

Primer combinations using F3 (SEQ ID NO:3) as the upstream primer, although they produced the expected PCR product with the β7 and SV29 cell lysates, did not produce a target-specific band with Sp6-γ/KG-1a cell lysates. The vector used in the Sp6-γ producer cell line (LXSN) is based on the LNL6 vector. According to Fig. 1, the F3 primer has a two nucleotide mismatch with the LNL6 vector sequence at the crucial 3′ end of the oligonucleotide and this difference most likely prevents

annealing and extension of the target in the Sp6-γ cell lysate.

Ten of the most promising primer combinations from the initial PCR, according to the criteria presented above, were tested in a second PCR with an increase in the annealing temperature to 62°C. The increased annealing temperature substantially reduced the incidence and intensity of non-specific background bands. Two particular primer combinations, F1/B5 and F2/B5, produced very strong target-specific PCR products. However, the F2/B5 primer combination still produced two faint nonspecific bands in the KG-1a cell lysate (Å200 and 600 bp; data not shown). A primer titration experiment was conducted with both primer pairs and it was determined that the incidence of non-specific bands could be eliminated without having a negative impact on product formation by using the primers at a concentration of 0.2 0 μM (data not shown). In addition, increasing the annealing temperature to 64°C further reduced the incidence of nonspecific background bands. Although the increased T_M slightly reduced the sensitivity of the assay with respect to the F2/B5 primer combination (data not shown), sensitivity was not affected when using the F1/B5 primer combination. Consequently, the F1/B5 primer combination was chosen as the best universal primer pair and it is these primers which are used in all subsequent PCR assays for MMLV-based proviruses.

Example 2. Sensitivity and specificity of the F1/B5 Ψ primers.

The absolute sensitivity of the F1/B5 Ψ primer configuration was determined following the procedure described in Example 1, against a 2-fold dilution series of β7 (Ψ positive) cell lysates at 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 cell equivalents. "-"

designates the negative PCR buffer control. PCR amplification of this dilution series, shown in Fig. 3A, showed that the 40 cycle PCR procedure was able to detect a single transduced cell by amplification of the F1/B5 Ψ sequence. This result was repeated by performing PCR on single β7 cells deposited by FACS into individual wells of a Terasaki plate. The results verified that the F1/B5 Ψ primer combination had single cell detection sensitivity in a 40 cycle reaction at a frequency of 50% (4/8; data not shown). By comparison, the 250 bp β-globin PCR product was detectable at a level of 8% (1/12; data not shown).

The sensitivity of the F1/B5 Ψ primers when SV29 (Ψ positive) are in a background of KG-1a (Ψ negative) cells was determined. Bulk PCR controls were made by adding KG-1a cells to a two-fold dilution series of SV29 cells. Lysates contained 10% (lysate A), 5% (lysate B), 2.5% (lysate C), 1.3% (lysate D), 0.6% (lysate E), and 0.3% (lysate F) SV29 cells, respectively. Lysate "0" contains 100% KG-1a cells. The total number of cell equivalents (including KG-1a cells) per 25 μl of lysate is 2,500. These conditions simulated the conditions of a bulk PCR to estimate percent transduction immediately following a transduction protocol. The results are shown in Fig. 3B. Absolute number of cell equivalents for each dilution in the series was 2,500 cells. To obtain a dose response in product yield, this PCR was reduced to 30 cycles total. The assay was sensitive down to 0.6% positive cells (dilution E) for the SV29 dilution series.

Example 3. <u>Effect of Residual Methylcellulose on Assay Sensitivity</u>.

To determine the level of detection obtainable with the Ψ primers when multiple clonogenic colonies growing in methylcellulose are pooled and processed for PCR, β7

and KG-1a methylcellulose colonies were pooled and processed according to the following ratio: 4:1, 3:2, and 2:3 KG-1a:β7 colonies. The five colonies were harvested using a P-10 pipet set at 3.0 μl, rinsed in 200 μl of PBS, vortexed and centrifuged for 30 seconds at 10K RPM in a benchtop centrifuge (TOMY). PBS was removed and 100 μl of lysing buffer (Example 1) was added. Samples were incubated for 60 minutes at 60°C, then transferred to a 95°C heat block for 10-15 minutes. One-tenth (10 μl) of each lysate was used in the PCR. The results (Fig. 4A) show that a single transduced colony was detectable, even when present in a background of 4 non-transduced colonies.

In order to determine if residual methylcellulose has any effect on PCR sensitivity, cell lysates were made which simulate the conditions of a pooled clonogenic methylcellulose colony assay using β7 cells (Ψ positive) in a background of KG-1a cells (Ψ negative), with and without the addition of methylcellulose medium. Duplicate sets of standards were made up which included 500, 250, and 50 β7 cells per 1500 KG-1a cells in 200 μl of PBS. Samples in lanes 1-6 all contain 375 KG-1a cell equivalents; 15 μl of methylcellulose media was added to one set of standards after the colonies were picked but before the PBS was replaced with PCR lysing buffer. All samples were then processed as above. One-fourth (25 μl) of each lysate was used in the PCR. The results are shown in Fig. 4B. Samples in lanes 1 and 4 contain 125 β7 cell equivalents; lanes 2 and 5 contain 62.5 β7 cell equivalents; and lanes 3 and 6 contain 12.5 β7 cell equivalents. Samples in lanes 4-6 were processed with the addition of methylcellulose medium, while samples in lanes 1-3 were processed without the addition of methylcellulose medium. Results established no

significant loss of PCR sensitivity or specificity in the presence of trace amounts of methylcellulose.

Example 4. Direct lysate PCR from single methylcellulose colonies in a 96-well format.

A repeating pipetter was used to fill 0.2 ml thin-wall PCR tubes with 50 µl of cell lysing buffer (Example 1). Tubes were held in a 96-well format by a MicroAmp tray/retainer assembly (Perkin Elmer/Applied Biosystems). Single clonogenic colonies growing in methylcellulose were harvested using a P-10 pipet set at 3.0 µl and placed directly into the tubes containing cell lysing buffer; while picking colonies the tubes were kept on ice. Several tubes, or "wells", were reserved for PCR and/or transduction controls (e.g., Mock transduced colonies) and at least one tube contained lysing buffer only as a negative template control. The plate was then covered and transferred to either a specially designed heating block (USA Scientific Plastics) or a thermal cycler (PE 9600, Perkin Elmer/Applied Biosystems) where it was incubated for one hour at 60°C, followed by a 5-minute incubation at 95°C (10 minutes if heating block is used).

Following the cell lysis procedure, lysates were transferred to a second plate containing the PCR master mix. Ψ primers were used alone or multiplexed with primers which amplify a region of the human β-globin sequence, BG1, 5'-CAA-CTT-CAT-CCA-CGT-TCA-CC-3' (SEQ ID NO:9); BG2, 5'-GAA-GAG-CCA-AGG-ACA-GGT-AC-3' (SEQ ID NO:10). The β-globin primers were used at a concentration of 0.16 µM. The appropriate single-target or multiplex PCR master mix can be aliquoted into each well of a fresh 96-well plate, before the addition of the methylcellulose colony lysate, using a repeating

pipetter. The cell lysate was found to be efficiently transferred to the tubes containing the PCR master mix (on ice) using a multichannel pipetter and filter barrier pipet tips to prevent aerosol contamination. Once all lysates were transferred to the second plate, and the appropriate PCR controls added, the tubes were covered and the plate transferred to the thermal cycler for amplification.

The PCR products were analyzed by ethidium bromide visualization of agarose gels. DNA from agarose gels was transferred to Zeta-Probe GT blotting membranes (Bio-Rad) in 20X SSPE and hybridized overnight at 68°C with the Ψ product oligonucleotide probe BK29 (5'-CTT-CGG-GGG-CCG-TTT-TTG-TGG-CCC-GAC-CT-3') (SEQ ID NO:11) that was end-labeled with [γ^{32}P]-ATP using standard protocols (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, NY). Blots were rinsed briefly in 2X SSPE/0.1% SDS, followed by 2x30 minute washes at 68°C in 0.1X SSPE/0.1% SDS, and then exposed 18-hours to a phosphor screen (Molecular Dynamics).

Results. Fig. 5A is the ethidium bromide-stained gel of the single target PCR (Ψ only - top row) and multiplex PCR (Ψ and β-globin - bottom row) for single methylcellulose colonies harvested as described above. All β7 and SV29 colonies are positive for both the Ψ sequence and β-globin, whereas the single KG-1a colonies are PCR positive only for β-globin. There is a faint band in one of the KG-1a colonies that runs slightly above the 50 bp ladder. This smaller band, presumably the result of the increase in the incidence of primer dimerization when there is no specific primer template available, is the result of non-specific amplification (as shown in Fig. 5B) and is easily distinguishable from the 134 bp target-specific Ψ PCR product. Fig. 5B is an

18-hour exposure after hybridization of the southern blot with the end-labeled internal probe BK29. Only the Ψ specific PCR product remains hybridized to BK29 after standard high stringency wash conditions.

Example 5. Transduction of $CD34^+$ $Thy\text{-}1^+$ Lin^- Stem/Progenitor Cells.

One hundred thousand $CD34^+$ $Thy\text{-}1^+$ Lin^- stem/progenitor cells were sorted from cytokine mobilized human peripheral blood (FACStar Plus, Becton Dickinson) and incubated with 1.0 ml of retroviral (Moloney-based LMTNL vector) supernatant (DMEM media harvested from retroviral producer cells) containing 50 ng/ml LIF, 25 ng/ml IL-3, 50 ng/ml IL-6, and 4 μg/ml protamine sulfate (LIF, IL-3, IL-6 supplied by Sandoz Pharmaceuticals; protamine sulfate, Sigma Chemical Co.). For the Mock transduction, cells were incubated in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Hyclone Laboratories), 50 U/ml penicillin (JRH Biosciences), 50 μg/ml streptomycin (JRH Biosciences), 2 mM L-glutamine (JRH Biosciences) and the growth factors at the concentrations mentioned above. Cells were pelleted and fresh supernatant (or DMEM complete) was added after 24 and 48 hours. Transduction was terminated after 72 hours and cells were resuspended in PBS containing 1% human serum albumin (alpha-Therapeutic Corp.) at which point, 2.5×10^3 cells were added to 5 ml of methylcellulose medium (80% MethoCult H4230, Stem Cell Technologies) containing the following cytokines; 10 ng/ml c-kit ligand (R&D Systems), 25 ng/ml GM-CSF (Sandoz Pharm.), 25 ng/ml G-CSF (Amgen), 10 ng/ml IL-3 (Sandoz Pharm.), and 1.2 U/ml of rhEPO (R&D Systems). Cells were plated in four 35 X 10 mm tissue culture dishes at 1.1 ml/dish (Nunc, Inc.) and incubated at 37°C/5.0% CO_2 for 14 days after which single hematopoietic colonies were

picked according to the protocol above and one-fifth (10 μl) of the lysate from an individual colony was amplified using the F1/B5 Ψ primers. The 96 wells of the PCR plate contained: (a) 80 randomly picked colonies from the experimental transduction, (b) 10 colonies from the Mock transduction, (c) 3 colonies of SV29 cells (Ψ positive controls), (d) 1 colony of KG-1a cells (Ψ negative control), and (e) 2 wells of PCR lysing buffer (negative PCR control). Ψ positive samples were analyzed by Southern blot using the internal probe, BK29. For post-PCR analysis, 15μl of the reaction was loaded onto a 4% agarose gel and stained with ethidium bromide.

Results. Fig. 6A shows the positive colonies (numbered above the lane) as detected by ethidium bromide staining. Positive colonies were analyzed by Southern hybridization using the internal probe, BK29 (Fig. 6B). Southern blot analysis verified the specificity of the Ψ positive samples. Results indicated that 19% (15/80) of the cells clonogenic in methylcellulose medium were transduced, as determined by amplification of the Ψ PCR product, whereas none of the 10 mock colonies were positive, further indicating that this assay can be carried out in a 96-well format without aerosol contamination of negative samples by positive samples.

Example 6. TaqMan™ Fluorescence-based Post-PCR Detection Assay.

Single colonies of clonogenic cells from an experimental transduction were picked according to the protocol above and 10 μl of the lysate from an individual colony was amplified as above except that a 24-mer oligonucleotide probe (5′-CCG-TTT-TTG-TGG-CCC-GAC-CTG-AGT-3′)(SEQ ID NO:12), internal to the F1/B5 Ψ PCR product, was included in the PCR master mix at a final concentration of 0.2 μM, and the total reaction volume

was increased to 60 μl. The 24-mer fluorescent probe (TaqMan™, synthesized by Perkin-Elmer Corporation) was labeled at the 5′ end with the reporter dye molecule, TET (6-carboxy-tetrachloro-fluorescein; emission λ 538 nm), while the quencher dye molecule, TAMRA (6-carboxytetramethyl-rodamine; emission λ 582nm), was added to the 3′ end of the probe via a linker arm nucleotide (LAN). A phosphate was added to the 3′ end to prevent extension of the probe during the PCR. During each cycle of the PCR the 5′→3′ exonuclease activity of *Thermus aquaticus* DNA polymerase cleaves the probe (Holland et al. (1991) Proc. Natl. Acad. Sci. USA 88:7276; Lee et al. (1993) Nuc. Acids Res. 21:3761), thereby increasing the fluorescence of the reporter dye at the appropriate wavelength. The increase in fluorescence is proportional to the concentration of template in the PCR.

Following the standard PCR thermal cycler protocol, 10 μl of the reaction was visualized on a 4% agarose gel containing ethidium bromide (as above). The 96 sample tray/retainer assembly, containing the remaining reaction volume, was transferred to the LS-50B luminescence spectrometer plate reader (Perkin-Elmer Corporation), the fluorescence emission (at 488 nm excitation λ) of both the reporter and quencher dye was measured and the ratio, RQ for each sample (emission intensity of reporter dye divided by the emission intensity of the quencher dye) was calculated. The TaqMan™ software then calculated the ΔRQ for each sample: $\Delta RQ = RQ^{+}$ (PCR with target template) minus RQ^{-} (PCR without target template). A positive result is determined as any value greater than the threshold ΔRQ. The threshold ΔRQ is determined by calculating both the mean and standard deviation of the Reporter:Quencher ratios for all no template controls (RQ^{-}) and then multiplying the standard deviation by a multiplier as determined by a table of student's

t-distribution values. When 10 no template controls (Mock transduced colonies) are analyzed per plate, the standard deviation of the Reporter:Quencher ratio is multiplied by 4.781 to achieve the ΔRQ threshold above which all samples have a 99.95% confidence level of being a positive result. In addition, if the fluorescence of the quencher molecule for any one sample is less than the mean quencher fluorescence of the no template controls then that sample is tagged with a "Low signal" warning so as to alert the investigator to a potentially invalid Reporter:Quencher ratio.

Results. Figs. 7A and 7B show the results of an experiment to compare the sensitivity and specificity of an ethidium bromide stained gel versus the TaqMan™ fluorescence assay. Fig. 7A displays the results for the entire plate. Samples #1-80 represent single methylcellulose colonies from the experimental transduction, whereas samples #81-90 are single methylcellulose colonies of Mock transduced cells. The 96-well plate also includes 3 positive controls (single SV29 colonies; indicated by a "+" in the last column of the plate), a negative colony control (KG-1a), and two negative PCR buffer controls. Note that in all cases where there is a detectable ethidium bromide stained band of the appropriate size, the TaqMan™ assay reports a positive result (sample ΔRQ>threshold ΔRQ). There are even three cases (#'s 18, 27, 54) for which the TaqMan™ assay reports a positive result which is not detectable from loading 10 μl of the PCR product on an ethidium bromide stained gel (99.95% confidence level). Figure 7B shows a direct comparison of a subset of the samples (#55-90). The dashed line represents the threshold ΔRQ above which samples are positive at the 99.95% confidence level. TaqMan™ positive samples are indicated by shaded bars. Note that non-specific bands that are slightly

smaller than the 134 bp PCR product (sample #'s 59, 77), primer-dimers (visible as a band at the bottom of each lane), and other artifacts of the PCR are not positive with the TaqMan™ assay and attest to the specificity of the fluorescent probe. It should be noted, however, that for each of the four experiments 10 no-template controls were used to calculate the threshold ΔRQ. Since the threshold ΔRQ, and consequently a TaqMan™ positive sample, is a function of the statistical power of the assay, it is possible that analyzing fewer no-template controls could increase the threshold ΔRQ and reduce the ability to statistically distinguish the faint positive samples from the no-template controls.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SYSTEMIX, INC.

(ii) TITLE OF INVENTION: RAPID ASSAY TO ESTIMATE GENE TRANSFER INTO CELLS

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCAACCCTG GGAGACGTCC 20

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAACACCCGG CCGCAACCCT G 21

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCCGACCTG AGTCCAAAAA TC 22

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCGCGGCTT CGGTCCCAAA C 21

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCAGCAGACA AGACGCGCGG C 21

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCTACCAGAA CCACATATCC CTCC 24

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAAAAATTCA GACGGAGGCG GG 22

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGTCTCCTAC CAGAACCACA TATCC 25

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAACTTCATC CACGTTCACC 20

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGAGCCAA GGACAGGTAC 20

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTTCGGGGGC CGTTTTTGTG GCCCGACCT 29

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGTTTTTGT GGCCCGACCT GAGT 24

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTGGAACTG ACGAGTTCGG AACACCCGGC CGCAACCCTG GGAGACGTCC 50
CAGGGACTTC GGGGGCCGTT TTTGTGGCCC GACCTGAGTC CAAAAATCCC 100
GATCGTTTTG GACTCTTTGG TGCACCCCCC TTAGAGGAGG GATATGTGGT 150
TCTGGTAGGA GACGAGAACC TAAAACAGTT CCCGCCTCCG TCTGAATTTT 200
TGCTTTCGGT TTGGGACCGA AGCCGCGCCG CGCGTCTTGT CTGCTGCAGC 250
ATCGTTCTGT GTTGTCTCTG TCTGACTGTG TTTCTGTATT TGTCTGAGAA 300
TATGGGCCAG ACTGTTACCA CTCCCT 326

What is claimed is:

1. A method for detecting the transfer of a nucleotide sequence into a target cell by a vector containing said nucleotide sequence and a unique vector sequence, comprising:

(a) obtaining said target cell or progeny thereof to be tested;

(b) lysing said cell, wherein a cell lysate is produced;

(c) contacting said cell lysate with a pair of oligonucleotide primers corresponding to said unique vector sequence under conditions wherein any sequence in the cell complementary to said primers is amplified; and

(d) detecting said amplified sequence, wherein detection of said sequence is indicative of gene transfer.

2. The method of claim 1, wherein said vector is a Moloney-Murine leukemia virus (MMLV)-based vector.

3. The method of claim 1, wherein said unique sequence is a packaging sequence.

4. The method of claim 3, wherein said packaging sequence is the Ψ packaging sequence.

5. The method of claim 1 wherein said cell constitutes a plurality of cells.

6. The method of claim 5, wherein said plurality of cells constitutes a cell colony derived from a single target cell.

7. The method of claim 6, wherein said cell colony is a hematopoietic stem/progenitor cell colony.

8. The method of claim 1, wherein said nucleotide sequence is a gene.

9. The method of claim 1, wherein said pair of oligonucleotide primers corresponding to a segment of MMLV DNA are selected from a group consisting of primers having the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

10. The method of claim 9, wherein said pair of oligonucleotide primers are SEQ ID NO:1 and SEQ ID NO:8.

11. The method of claim 1, further comprising contacting said cell lysate with a second pair of oligonucleotide primers corresponding to a marker DNA sequence of interest.

12. The method of claim 11, wherein said second pair of oligonucleotide primers correspond to β-globin and have the sequences of SEQ ID NO:9 and SEQ ID NO:10.

13. The method of claim 1, wherein said detection is by gel electrophoresis.

14. The method of claim 1, wherein said amplified DNA product is detected by fluorescence.

15. A direct lysate method for detecting Moloney-Murine leukemia virus (MMLV)-based gene transfer into a target cell, comprising:

(a) obtaining a cell to be tested;

(b) lysing said cell, wherein a cell lysate is produced;

(c) contacting said cell lysate with a pair of oligonucleotide primers corresponding to a nucleotide sequence of MMLV DNA under conditions wherein a DNA sequence complementary to said primers is amplified, and wherein said primers have the sequence of SEQ ID NO:1 and SEQ ID NO:8; and

(d) detecting said amplified DNA sequence, wherein detection of said DNA sequence is indicative of gene transfer.

16. A fluorescent method for detecting Moloney-Murine leukemia virus (MMLV)-based gene transfer into a target cell, comprising:

(a) obtaining a cell to be tested;

(b) lysing said cell, wherein a cell lysate is produced;

(c) contacting said cell lysate with a pair of oligonucleotide primers corresponding to a sequence of MMLV DNA under conditions wherein a oligonucleotide sequence complementary to said primers is amplified, and a fluorescent probe complementary to a portion of said amplified DNA, wherein the primers are specific to a portion of a packaging sequence;; and

(d) detecting the fluorescence in said cell lysate, wherein the level of fluorescence is proportional to the amount of amplified DNA sequence present, and wherein detection of fluorescence over control values is indicative of gene transfer.

17. The method of claim 16, wherein said pair of oligonucleotide primers have the sequence of SEQ ID NO:1 and SEQ ID NO:8.

18. The method of claim 1, wherein said method is conducted with multiple cell colonies in a multi-well plate.

19. Oligonucleotide primers useful in the method of claim 1, said primers selected from the group consisting of primers having the sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

20. The oligonucleotide primers of claim 19, wherein said primers have the sequences of SEQ ID NO:1 and SEQ ID NO:8.

21. A kit for detecting gene transfer by MMLV-based vectors, comprising:

a primer pair selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8; and

instructions for using the kit components for detecting gene transfer by MMLV-based vectors in a cell according to the method of claim 1.

22. The kit of claim 21 further comprising:

PCR lysing buffer, dNTPs, polymerase, a well tray, and detection reagents.

```
                    10        20        30   F1   40        50
McMuLV:  GGTGGAACTGACGAGTTCGGAACACCCGGCCGCCAACCCCTGGGAGACGTCC
LNL6/LYSN: ..........................................
MPG-LacZ:  ..........................................

                    60        70        80        90       100
McMuLV:  CAGGGACTTCGGGGGCCGTTTTGTGGCCCGACCTGAGTCCAAAAATCCC
LNL6/LYSN: ..........T.....................G----------GGGA
MPG-LacZ:  ..........................................

                   110       120       130       140   B5  150
McMuLV:  GATCGTTTTGGACTCTTTGGTGCACCCCCCTTAGAGGAGGGATATGTGGT
LNL6/LYSN: -----A-G----------GA----G-C----------..........
MPG-LacZ:  ..........................................

                   160       170       180       190       200
McMuLV:  TCTGGTAGGAGACGAGAACCTAAAACAGTTCCCGCCTCCGTCTGAATTTT
LNL6/LYSN: ..........................................
MPG-LacZ:  ..........................................

                   210       220       230       240       250
McMuLV:  TGCTTTCGGTTTGGGACCGAAGCCGCGCGCCGTCTTGTCTGCTGCAGC
LNL6/LYSN: ..........A...........----------..........
MPG-LacZ:  ..........................................

                   260       270       280       290       300
McMuLV:  ----------ATCGTTCTGTGTTGTCTCATGTCTGACTGTGTTTCTGTATTTG
LNL6/LYSN: GGTGCAGC..........................................
MPG-LacZ:  ----------..........................................

                   310       320       330       340
McMuLV:  TCTGAGAATATGGG----------CCAGACTGTTACCACTCCCT
LNL6/LYSN: .....A.....TA----------..........
MPG-LacZ:  ..........CCCGGG..........
```

FIG. 1

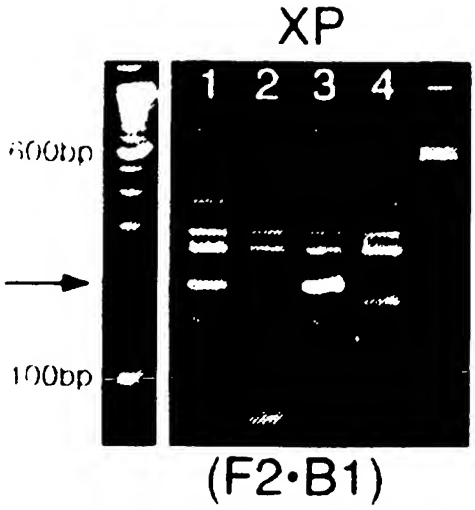

FIG. 2A

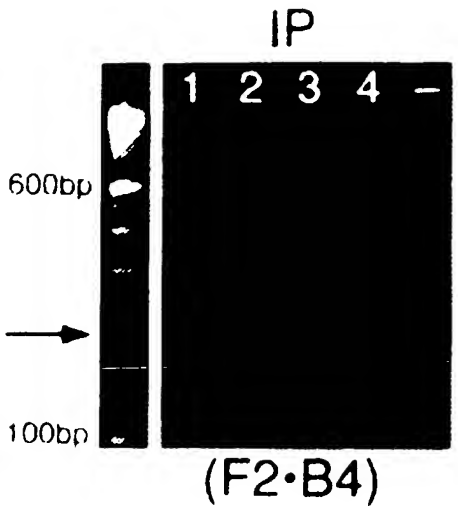

FIG. 2B

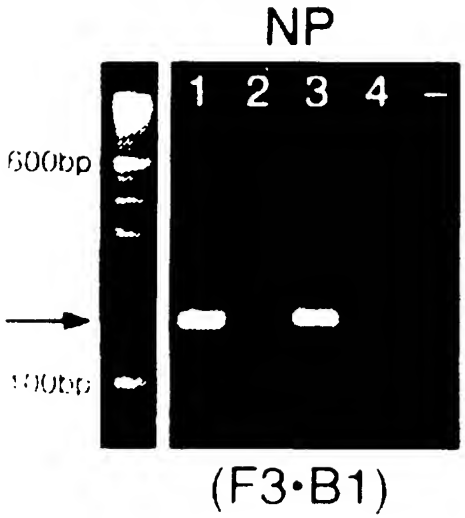

FIG. 2C

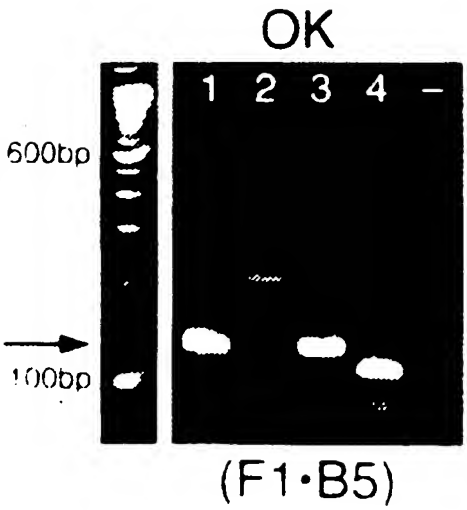

FIG. 2D

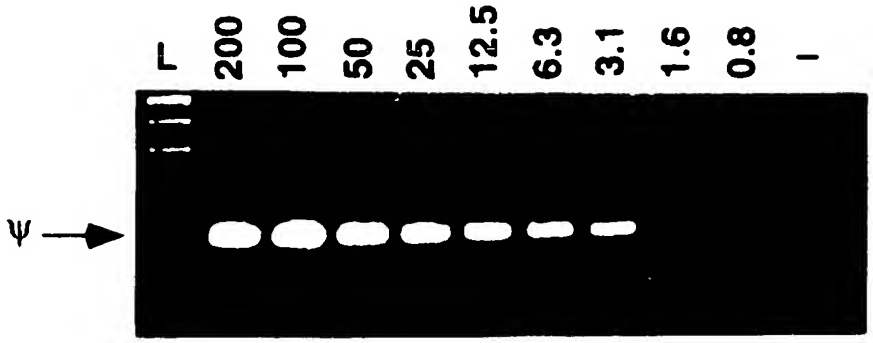

FIG. 3A

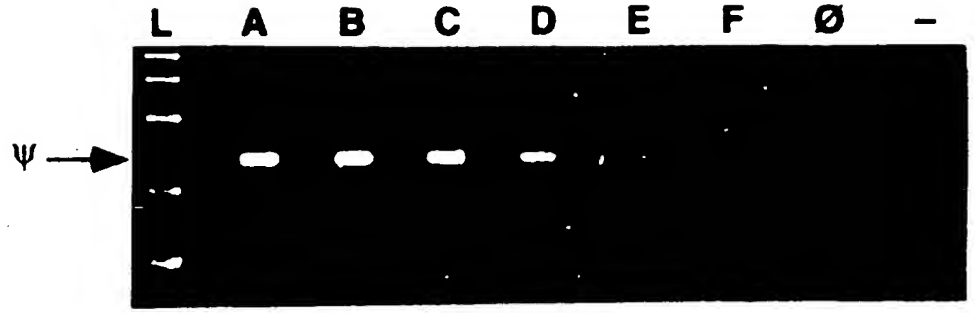

FIG. 3B

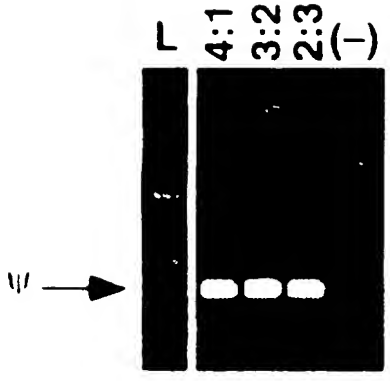

FIG. 4A

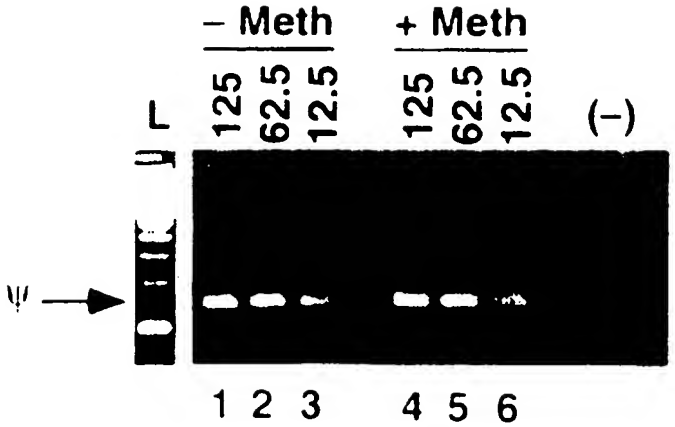

FIG. 4B

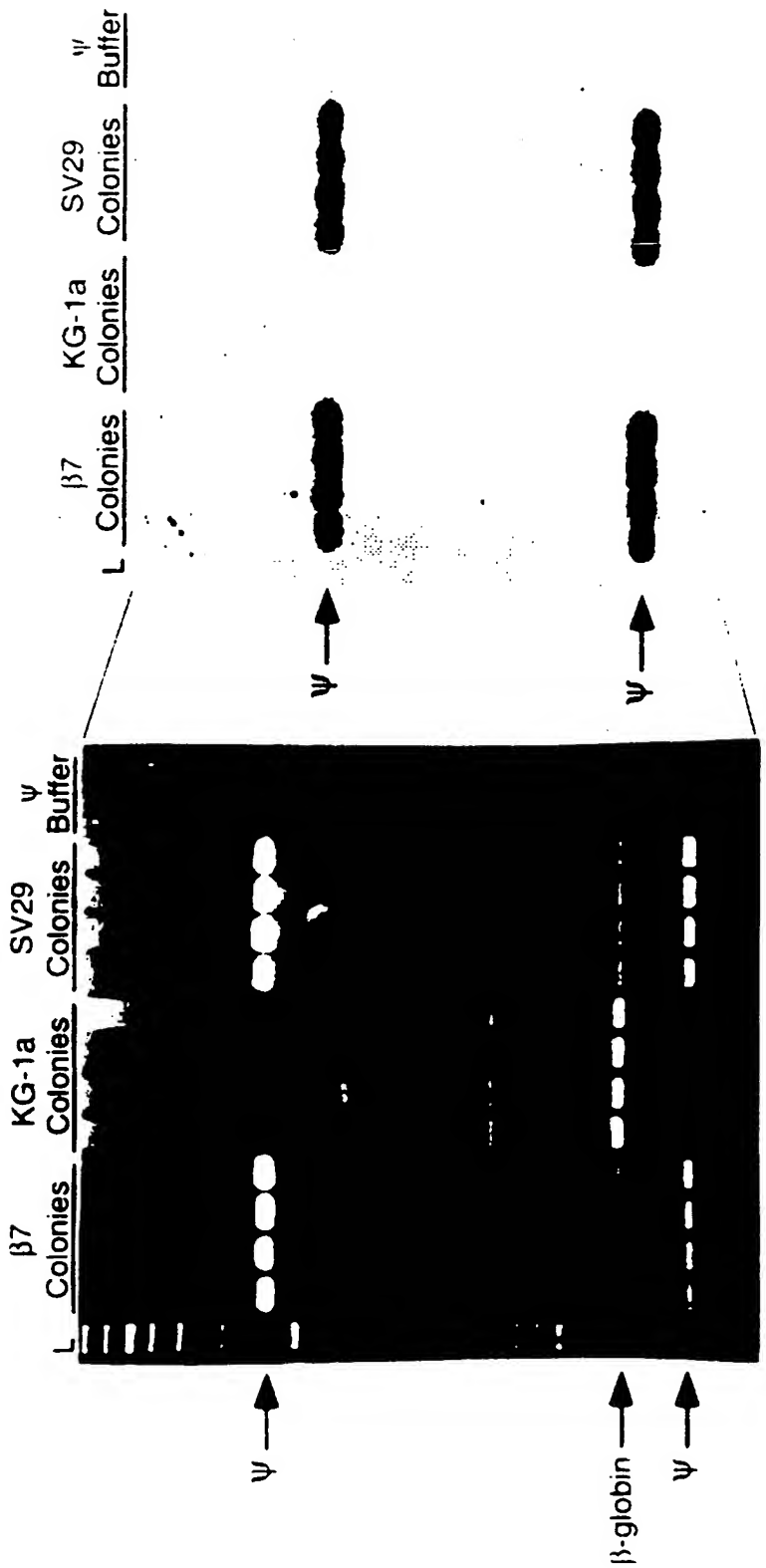
L
β7 Colonies
KG-1a Colonies
SV29 Colonies
ψ Buffer
ψ
β-globin
ψ
ψ

FIG. 5A

L
β7 Colonies
KG-1a Colonies
SV29 Colonies
ψ Buffer
ψ
ψ

FIG. 5B

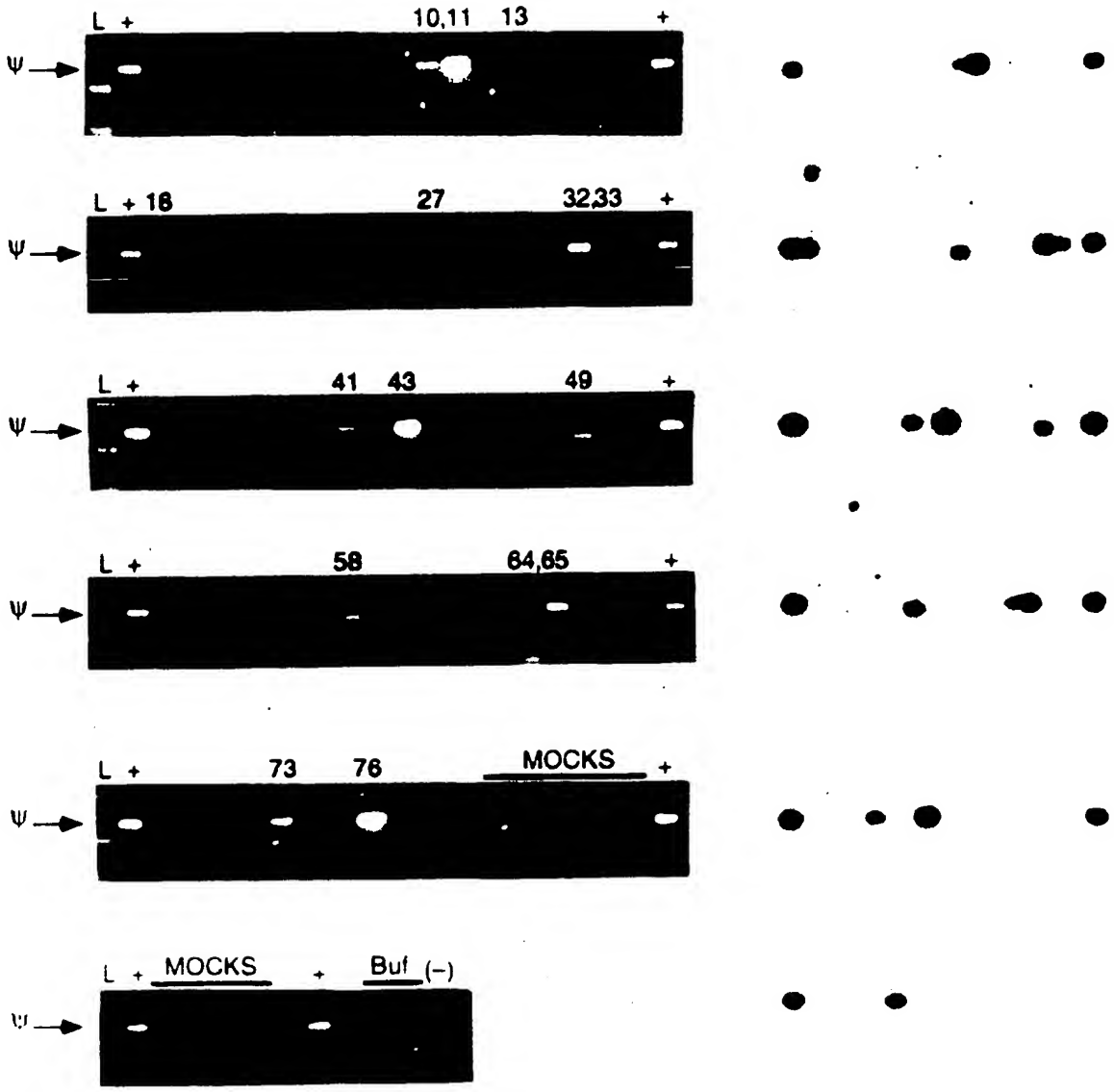
L +
10,11
13
+
Ψ
L + 16
27
32,33 +
Ψ
L +
41
43
49
+
Ψ
L +
58
64,65
+
Ψ
L +
73
76
MOCKS
+
Ψ
L +
MOCKS
+
Buf (-)
Ψ

FIG. 6A

FIG. 6B

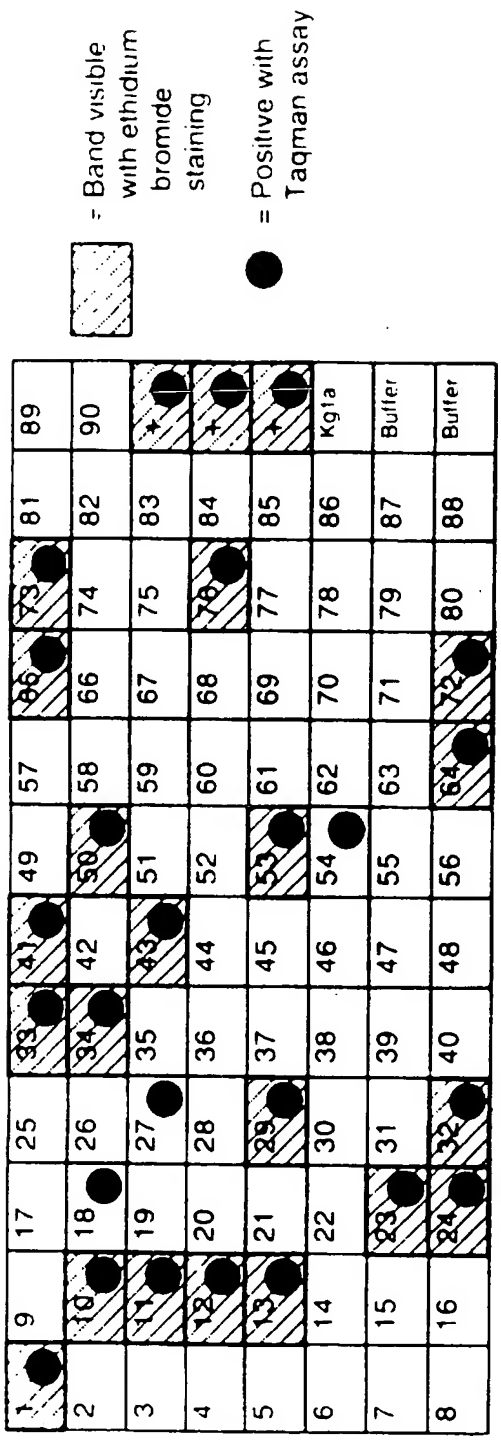
= Band visible with ethidium bromide staining
= Positive with Taqman assay
Kg1a
Buffer
Buffer

FIG. 7A

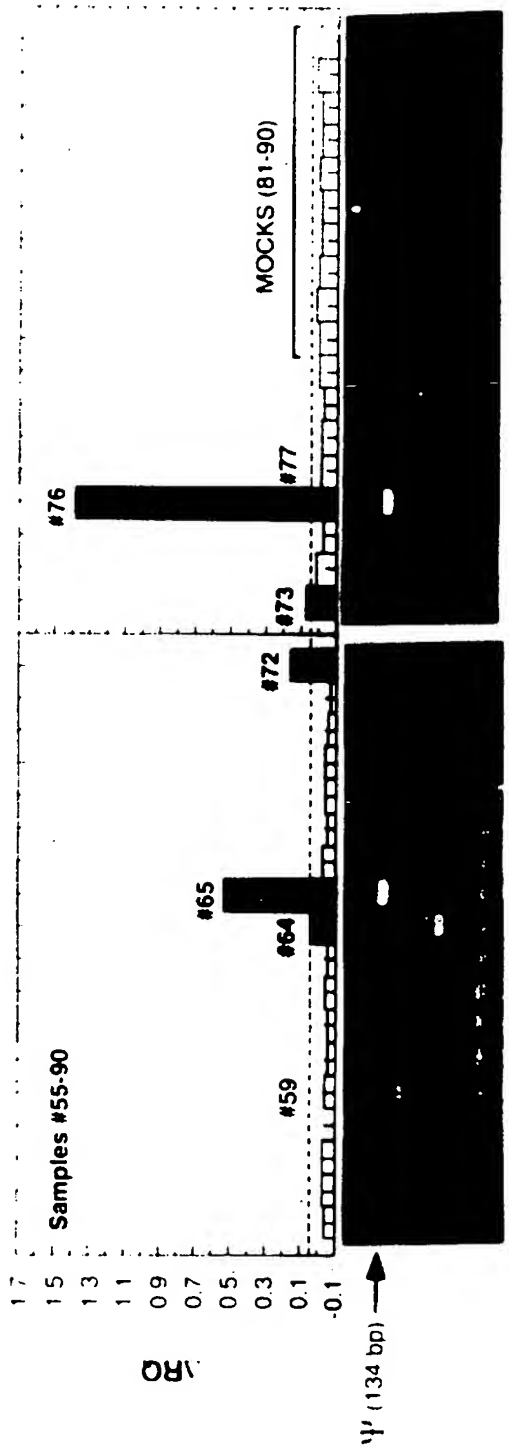
Samples #55-90
MOCKS (81-90)
#59
#64
#65
#72
#73
#76
#77
ΔRQ
Ψ (134 bp)

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19495

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68, 1/70; C12P 19/34; A61K 48/00; C07H 21/04, 21/02
US CL :435/6,5, 91.2; 514/44; 536/24.5,24.3, 24.33, 23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,5, 91.2; 514/44; 536/24.5,24.3, 24.33, 23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	COURNOYER, D. et al. Gene Transfer of Adenosine Deaminase into Primitive Human Hematopoietic Progenitor Cells. Human Gene Therapy. 1991, Volume 2, pages 203-213, especially page 205, and Figure 1.	1-22
Y,P	US, 5,545,528 A (MITSUHASHI et al.) 13 August 1996, see entire document.	1-22
Y,P	US, 5,512,441 A (RONAI) 30 April 1996, column 6, lines 50-60.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 FEBRUARY 1997	Date of mailing of the international search report 23 APR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIANNE REES Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19495

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, BIOBUSINESS, CABA, CAPLUS, CANCERLIT, DGENE, DRUGU, EMBASE, GENBANK, JAPIO, MEDLINE, USPATFULL, WPIDS
search terms: vector sequences, PCR or polymerase chain reaction,, and transfer, transfect or transform, moloney murine leukemia virus or MMLV, gene therapy, gene targeting, gene disruption, SEQ. ID. Nos.